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The Molecular Motor KIF1A Transports the TrkA Neurotrophin Receptor and Is Essential for Sensory Neuron Survival and Function

Highlights

- KIF1A haploinsufficiency caused sensory neuronal defects in mice
- TrkA(+) nociceptive neurons were specifically decreased in *Kif1a*$^{+/-}$ DRGs
- KIF1A specifically transports the NGF receptor TrkA to enhance PI3K signaling
- In surviving neurons, PI3K-dependent sensitization of the pain receptor was impaired

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In Brief

How does the kinesin molecular motor contribute to the sensory nervous system? Using mouse molecular genetics, Tanaka et al. identified that KIF1A specifically transports the NGF receptor TrkA into axons of DRG neurons and that it serves in PI3K-mediated cell survival and sensitization.

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The Molecular Motor KIF1A Transports the TrkA Neurotrophin Receptor and Is Essential for Sensory Neuron Survival and Function

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SUMMARY

KIF1A is a major axonal transport motor protein, but its functional significance remains elusive. Here we show that KIF1A-haploinsufficient mice developed sensory neuropathy. We found progressive loss of TrkA(+) sensory neurons in *Kif1a*$^{+/-}$ dorsal root ganglia (DRGs). Moreover, axonal transport of TrkA was significantly disrupted in *Kif1a*$^{+/-}$ neurons. Live imaging and immunoprecipitation assays revealed that KIF1A bound to TrkA-containing vesicles through the adaptor GTP-Rab3, suggesting that TrkA is a cargo of the KIF1A motor. Physiological measurements revealed a weaker capsaicin response in *Kif1a*$^{+/-}$ DRG neurons. Moreover, these neurons were hyposensitive to nerve growth factor, which could explain the reduced neuronal survival and the functional deficiency of the pain receptor TRPV1. Because phosphatidylinositol 3-kinase (PI3K) signaling significantly rescued these phenotypes and also increased *Kif1a* mRNA, we propose that KIF1A is essential for the survival and function of sensory neurons because of the TrkA transport and its synergistic support of the NGF/TrkA/PI3K signaling pathway.

INTRODUCTION

Cells receive extracellular information through surface receptors, which are synthesized at the endoplasmic reticulum, modified at the Golgi apparatus, transported toward the plasma membrane, and presented to the cell surface. These vesicles bud off from the *trans*-Golgi network and are transported by microtubule- and ATP-dependent molecular motors such as kinesin superfamily proteins (KIFs) (De Matteis and Luini, 2008; Hirokawa et al., 2009, 2010; Hirokawa and Takemura, 2005; Nakata et al., 1998; Sciaky et al., 1997; Verhey and Hammond, 2009). Mammals express 45–46 KIFs (Lawrence et al., 2004; Miki et al., 2001), and KIF1A is a microtubule plus end-directed motor protein that belongs to the kinesin-3 family and is involved in the anterograde transport of synaptic vesicle precursors (Okada et al., 1995) supported by the GTP-Rab3 adaptor (Niwa et al., 2008). A genetic study demonstrated that *Kif1a*$^{-/-}$ mice die shortly after birth, with a reduced number of synaptic vesicles and a significant loss of neurons (Yonekawa et al., 1998). The relationship between cell death and the function of KIF1A remains elusive. In addition, the function of KIF1A in mature animals is unknown.

Pain and heat sensations are transduced through primary afferent nociceptors (PANs), which are pseudo-unipolar neurons located in the dorsal root ganglia (DRGs). The branches of the axon innervate both the peripheral tissues and the dorsal root of the spinal cord (Kandel, 2013). The peptidergic population of PANs coexpresses the following molecules: the neurotransmitter substance P (SubP); tropomyosin receptor kinase A (TrkA), which is a high-affinity tyrosine kinase receptor specific for nerve growth factor (NGF); and transient receptor potential cation channel subfamily V1 (TRPV1), which is a heat- and capsaicin-evoked cation channel (Caterina et al., 1997; Hökfelt et al., 1975; Tominaga et al., 1998; Verge et al., 1992). Genetic ablation of the *Trpv1* gene results in severe sensory loss (Caterina et al., 2000). TRPV1-F11 cells serve as a good in vitro model system of PANs (Nakanishi et al., 2010).

NGF and TrkA are major modulators of PANs, not only for survival but also for sensitization. Knockout mice for NGF, TrkA, and the TrkA transcription factor *Klf7* exhibit extensive loss of DRG neurons and fail to respond to nociceptive stimuli (Crowley et al., 1994; Lei et al., 2005; Smeyne et al., 1994). TRPV1 expression levels as well as TRPV1 phosphorylation levels are controlled by the TrkA neurotrophin receptor (Amaya et al., 2004; Ji et al., 2002; Xue et al., 2007; Zhang et al., 2005). Previous studies have shown that the sensitivity of TRPV1 is stimulated by TrkA signaling. The signaling supports TRPV1 expression and phosphorylation, and tyrosine phosphorylation sensitizes TRPV1 (Amaya et al., 2004; Ji et al., 2002; Xue et al., 2007; Zhang et al., 2005). The binding of NGF to TrkA leads to activation of the Ras and phosphatidylinositol 3-kinase (PI3K) signaling pathways (Ohmichi et al., 1992; Soltoff et al., 1992; Thomas et al., 1992). NGF is secreted from many types of peripheral cells and binds to TrkA at the axon terminal

(Delcroix et al., 2003; Saxena et al., 2005; Ye et al., 2003). It is fundamental for neuronal viability and also serves as an important mediator of inflammatory pain (McMahon, 1996). Therefore, the mechanism by which TrkA is transported to axonal terminals appears to be fundamental for normal neuronal function and survival as well as for pathological hyperalgesia because of inflammatory responses (Sofroniew et al., 2001). Indeed, previous studies have shown that TrkA bidirectionally moves in DRG neuronal axons and that the retrograde motor for TrkA is cytoplasmic dynein (Deinhardt et al., 2006; Nakata et al., 1998; Saxena et al., 2005). However, the mechanisms of TrkA anterograde axonal transport and its physiological relevance remain largely unknown.

In this study, we show that KIF1A-haploinsufficient (*Kif1a*$^{+/-}$) mice suffered from sensory neuropathy with a significant and specific reduction in the function and number of TrkA neurons. Previous studies have shown that the sensitivity of TRPV1, a capsaicin receptor, is stimulated by TrkA signaling (Amaya et al., 2004; Ji et al., 2002; Xue et al., 2007; Zhang et al., 2005) in inflammatory hyperalgesia. We show evidence that KIF1A transports TrkA and is essential for the NGF/TrkA/Ras/PI3K signaling pathway that augments TRPV1 tyrosine phosphorylation and cell surface presentation, which plays an important role in pain sensation. Therefore, the anterograde axonal transport of TrkA mediated by KIF1A is essential for sensory neuronal function, possibly by synergistically supporting neuronal viability and modulating TRPV1 channels.

RESULTS

KIF1A Is Expressed in DRGs, and *Kif1a*$^{+/-}$ Mice Develop Sensory Neuropathy

Kif1a knockout mice were generated using Cre/*loxP*-mediated gene targeting (Figure S1), with a single frameshift introduced by deleting exons P to P + 2. To measure KIF1A expression in heterozygotes, anti-KIF1A antibodies were newly prepared, and immunoblot analysis was performed using α-tubulin as a loading control (Figures 1A and 1B). The results demonstrated that KIF1A expression in the 10-month-old *Kif1a*$^{+/-}$ mouse brain was approximately half of that in the *Kif1a*$^{+/+}$ mice, although the expression of KIF1Bβ, a highly homologous motor of KIF1A (Niwa et al., 2008; Zhao et al., 2001), was largely unchanged. In adult DRGs or sensory ganglia, KIF1Bβ was expressed at only trace levels, and the decrease in KIF1A protein levels in the *Kif1a*$^{+/-}$ DRGs appeared to be more severe than that in the *Kif1a*$^{+/-}$ brains. Because the KIF5B levels remained unchanged, the result was considered to be specific.

In our behavioral analyses, we noticed that sensory responses were affected in the *Kif1a*$^{+/-}$ mice (Figures 1C–1F). First, the von Frey test was used to measure the nociceptive response (Figure 1C; Pitcher et al., 1999; Fuchs et al., 1999; Crawley, 2007). A longitudinal study conducted over 3–6 months of age suggested that the sensitivity of the *Kif1a*$^{+/-}$ mice to poking by von Frey hair was significantly impaired after 5–6 months of age. Because this phenotype was more obvious in older mice, 7- to 12-month-old mice were utilized, respectively, for the formalin test in two phases (Figure 1D): the hot water tail flick test at 47.5°C (Figure 1E) and the hot plate test at 52.5°C (Figure 1F). These behavioral tests consistently demonstrated diminished sensory responses, suggesting that the *Kif1a*$^{+/-}$ mice progressively developed sensory defects for nociceptive stimuli.

The Number of TrkA(+) Neurons Is Specifically Reduced in *Kif1a*$^{+/-}$ DRGs

Previous studies have established that heat and mechanical stimulation are mainly received by TrkA(+) neurons in the DRG (Amaya et al., 2004; Chuang et al., 2001; Einarsdottir et al., 2004; Indo et al., 1996; Ji et al., 2002; Zhang et al., 2005). Thus, TrkA(+) and TrkB(+) neurons in the DRG were visualized by immunostaining. The results reproducibly demonstrated that the percentage of TrkA(+) neurons, but not that of TrkB(+) neurons, was significantly reduced in 20-month-old *Kif1a*$^{+/-}$ mice (Figures 2A and 2B). This difference tended not to be detected at 2 months of age but was found at 9 months of age (Figure 2C), suggesting that the effects of KIF1A reduction are cell type-specific and progressive.

To test whether this specific loss of TrkA neurons correlates with cell death, we used the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to detect apoptosis in mice of various ages (Figures 2D and 2E). At 8 months of age, the *Kif1a*$^{+/-}$ DRGs exhibited a higher level of apoptosis than the *Kif1a*$^{+/+}$ DRGs, consistent with the stage at which the significant population change first occurred (Figure 2C). However, at 5 months of age, there was a discrepancy between the behavioral phenotype (Figure 1C) and the observation of a low rate of apoptosis (Figure 2E), suggesting that the initial impairment of sensory function at this stage was barely due to neuronal loss and was instead mainly due to the functional deficit of the PANs.

The Capsaicin Response Is Reduced in *Kif1a*$^{+/-}$ DRG Neurons

Although the number of TrkA(+) neurons was significantly reduced in the *Kif1a*$^{+/-}$ mice, a certain population of TrkA(+) sensory neurons was still alive. Thus, whether these surviving neurons were functionally normal remained an open question. Capsaicin is a well characterized compound that produces a sensation of pain and stimulates TRPV1 channels, which are coexpressed with TrkA on the surface of DRG nociceptive neurons (Caterina et al., 1997; Kim et al., 2008; Tominaga et al., 1998).

To test this property of living sensory neurons in *Kif1a*$^{+/-}$ mice, Ca imaging of dissociated DRG neurons from mice at 9 months of age was first performed (Figures 3A and 3B). We stimulated neurons with 1 μM capsaicin in complete medium containing NGF and measured the Ca transients using confocal laser-scanning microscopy. Capsaicin elevated the intracellular Ca ($[Ca]_i$) level in a population of primary cultured DRG neurons to some extent, but the amplitude of this Ca elevation was significantly reduced in *Kif1a*$^{+/-}$ neurons. Because overnight treatment with the TRPV1 channel blocker SB705498 (1 μM) almost completely abolished the Ca elevation in the *Kif1a*$^{+/+}$ neurons (Figures 3A and 3B, brown graphs), these data indicate that KIF1A deficiency resulted in TRPV1 channel inactivation in DRG nociceptive neurons.

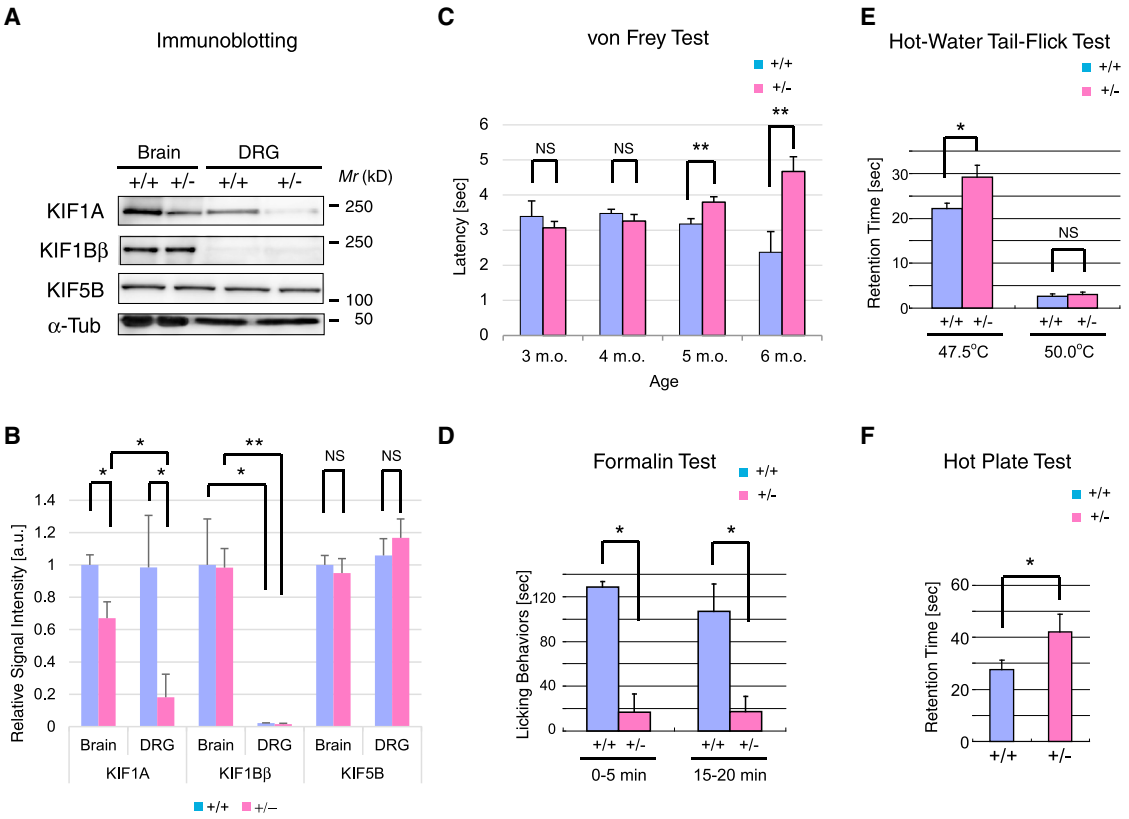


Figure 1. Impairment of the Sensory Function of *Kif1a*$^{+/-}$ Mice

(A and B) Immunoblot analysis against the molecular motors KIF1A, KIF1Bβ, and KIF5B in 10-month-old (m.o) *Kif1a*$^{+/+}$ *and Kif1a*$^{+/-}$ mouse brains and DRGs (A) and the corresponding quantification (B). α-Tub, α-tubulin as a sample loading control. n = 3–6. NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; Welch's t test. Error bars show SEM. See also Figure S1 for knockout mouse generation.

(C–F) Behavioral tests of sensory functions. NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; Welch's t test. Error bars show SEM.

(C) von Frey test at 3–6 months of age (n = 5–14). Shown is the latency for hind paw flick following poking by a von Frey hair.

(D) Formalin test at 9–10 months of age (n = 3). Time spent licking the paw or biting was recorded for 5 min during two response phases following intraplantar injection.

(E) Hot water tail flick test at 8–10 months of age (n = 6–35). Shown is the latency of tail withdrawal when the tail was immersed in a beaker of water at the indicated temperature.

(F) Hot plate test at 7–10 months of age (n = 10 and 11). Shown is the latency to escape from a plastic cylinder placed on a 52.5°C hot plate.

In addition, whole-cell patch-clamp recordings of dissociated DRG neurons were performed in conjunction with capsaicin stimulation. They were voltage-clamped at a holding potential of –60 mV, and 5 μM capsaicin was applied to the cells in the presence of NGF. The inward current of the capsaicin response of *Kif1a*$^{+/+}$ neurons was similar to that observed previously (Kim et al., 2008), but that in the *Kif1a*$^{+/-}$ neurons was significantly weaker (Figure 3C, left column). When NGF was depleted from the culture medium, these responses were significantly reduced to a similarly lower level (Figure 3C, right column), as expected from NGF dependence of TRPV1 activity (Crowley et al., 1994; Lei et al., 2005; Smeyne et al., 1994). Accordingly, the current density in the complete medium was 103.5 ± 17.7 pA/pF and 70.6 ± 12.0 pA/pF in the *Kif1a*$^{+/+}$ and *Kif1a*$^{+/-}$ neurons, respectively (Figure 3D; mean ± SD, $p < 0.01$, Student's t test, n = 7). These results suggest the presence of NGF-dependent functional deficits of the capsaicin receptor in *Kif1a*$^{+/-}$ sensory neurons.

Distribution of TRPV1 and TrkA Were Differentially Altered by KIF1A Deficiency

We then compared the expression of TRPV1 and TrkA receptor channels in the distal axons of primary cultured DRG nociceptive neurons using immunofluorescence microscopy (Figures 4A and 4B). First, the TrkA signal in the *Kif1a*$^{+/-}$ distal axon was significantly reduced. The localization of TRPV1 there tended to be more punctate, perhaps included in cytoplasmic endosome-like structures, but its total level in the distal axon remained unchanged. However, when the surface proteins were specifically labeled by omitting the permeabilization step of the staining, the expression of both of these proteins was revealed to be significantly reduced on the surface of the *Kif1a*$^{+/-}$ axons, consistent with the Ca and electrophysiology measurements (Figure 3).

This significant reduction of TrkA and TRPV1 from the KIF1A-deficient cell surface was biochemically confirmed using a cell surface biotinylation assay in a knockdown system of TRPV1-F11 cells (Nakanishi et al., 2010). The transduction of knockdown

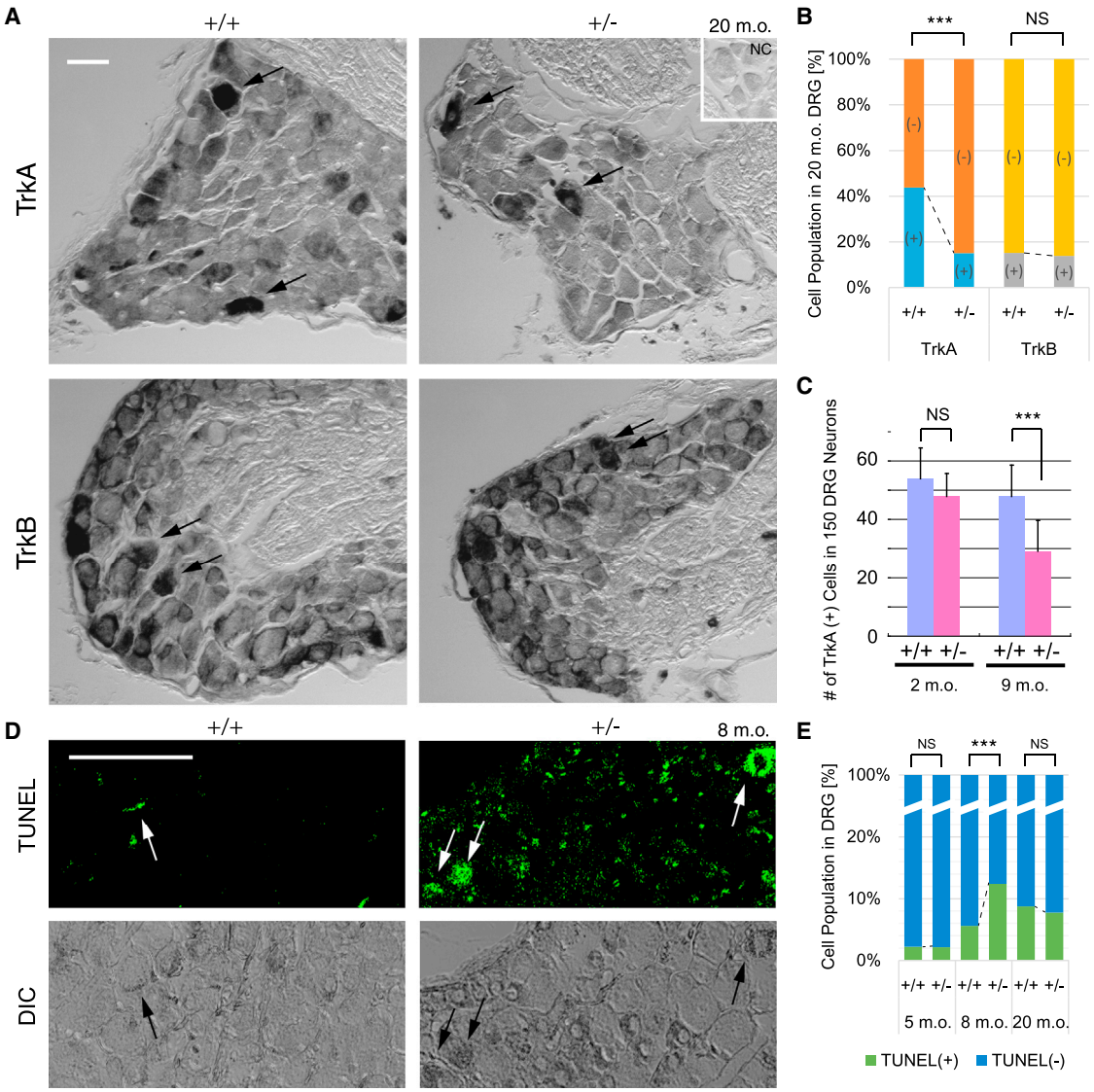


Figure 2. Immunohistochemical Analysis of DRG Neurons

(A–C) Immunohistochemistry (A) and the corresponding quantification (B and C) for TrkA and TrkB in DRGs of 20-month-old (m.o.) (A and B) and younger (C) mice of the indicated genotypes. Prox, proximal; Dist, distal. Inset, negative control (NC) with normal rabbit IgG. Scale bar, 10 μm. NS, $p > 0.05$; ***$p < 0.001$, chi-square test (B) and Student's t test (C). n = 432–899 (B) and 8 DRG sections from 4 *Kif1a*$^{+/+}$ mice and 4 *Kif1a*$^{+/-}$ mice (C), respectively. Error bars show SD. Note that the TrkA(+) but not the TrkB(+) population (arrows) is specifically reduced among *Kif1a*$^{+/-}$ DRG neurons older than 9 months.

(D and E) TUNEL assay of DRG sections of the indicated genotypes (D) and quantification of the results for various stages (E). Note that the apoptotic cell population is specifically elevated in *Kif1a*$^{+/-}$ DRGs at 8 months of age. n = 582–1549 cells in 3–8 DRG sections from 2 mice, respectively. NS, $p > 0.05$; ***$p < 0.001$, chi-square test. Scale bar, 100 μm.

microRNA (miRNA) for the mouse *Kif1a* gene specifically reduced the KIF1A expression level compared with that of the scrambled negative control (Figures 4C and 4D). The cell surface levels of TrkA and TRPV1 in the KIF1A knockdown cells were significantly reduced compared with those in the control cells.

We further conducted a nerve ligation assay in living animals to assess the cumulative axonal transport rate in saphenous nerves of 9-month-old mice (Hirokawa et al., 1990; Perlson et al., 2009). 5 hr after ligation, a 5-mm length of the proximal and distal nerve was subjected to immunoblot analysis (Figure 4E and F). With using β3-tubulin (TUBB3) as a loading control, the proximal-to-distal ratio of accumulated KIF5A in both genotypes was similar to that in a previous report (Perlson et al., 2009), supporting the validity of this experiment. However, the proximally accumulated amount of TrkA, but not that of the capsaicin receptor TRPV1, was significantly reduced in the *Kif1a*$^{+/-}$ nerves. Because TRPV1 and TrkA are largely coexpressed in nociceptor neurons (Caterina et al., 1997; Hökfelt et al., 1975; Tominaga et al., 1998; Verge et al., 1992), these results more likely suggest that

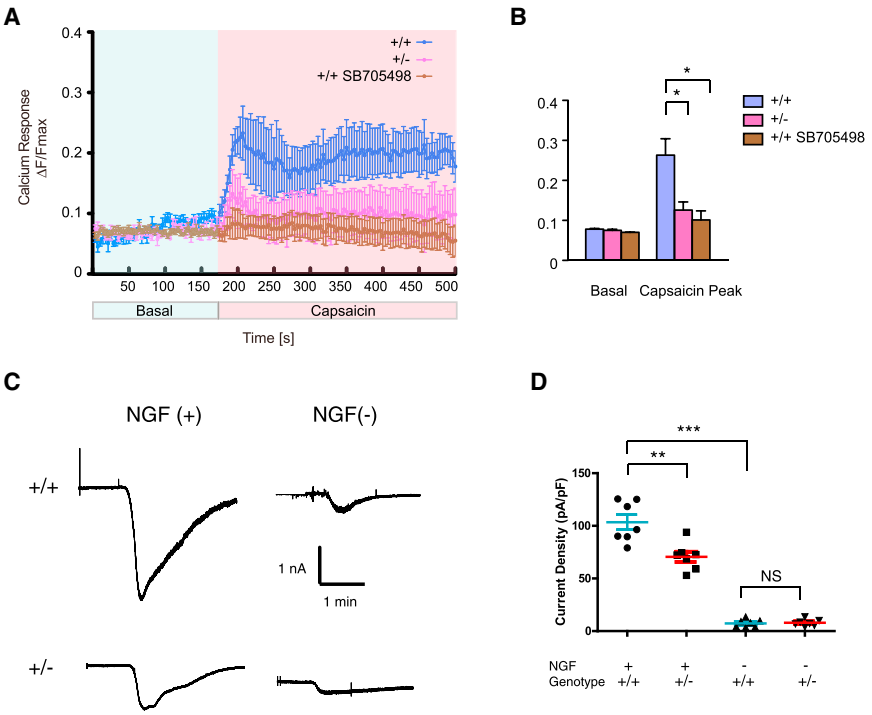


Figure 3. KIF1A and NGF Are Essential for TRPV1 Activity

(A and B) Ca imaging of primary DRG neurons from 9-month-old male mice stimulated with 1 μM capsaicin.

(A) Representative Ca transients of the indicated genotypes probed by Fluo-4AM. Note that the TRPV1 inhibitor SB705498 (1 μM overnight) almost completely eliminated the Ca response in wild-type cells, which was significantly impaired by the reduction of KIF1A. Scale bar, 10 μm.

(B) Quantification of the peak amplitudes. *$p < 0.05$, one-way ANOVA. n = 11–16 from 3 mice each. Error bars show SEM.

(C and D) Electrophysiology of primary DRG neurons stimulated with 5 μM capsaicin.

(C) Representative membrane currents in patch-clamp recordings of cultured *Kif1a*$^{+/+}$ and *Kif1a*$^{+/-}$ DRG neurons isolated from 9-month-old male mice are shown in the presence (+) or absence (–) of 200 ng/ml NGF. Cells were voltage-clamped at –60 mV. Calibration, 1 nA and 1 min.

(D) Current densities in DRG neurons of the indicated genotypes following stimulation with 5 μM capsaicin in the presence or absence of NGF. NS, $p > 0.05$; **$p < 0.01$; ***$p < 0.001$; Welch's t test. n = 7. The scatterplot was overlaid on the mean ± SEM. Note that the NGF-invoked enhancement of the current intensity in the *Kif1a*$^{+/-}$ neurons was significantly smaller than that in the *Kif1a*$^{+/+}$ neurons.

anterograde axonal transport of TrkA was specifically reduced in the *Kif1a*$^{+/-}$ peripheral nerves.

Axonal Transport of TrkA Is Reduced in *Kif1a*$^{+/-}$ Neurons

The axonal transport of enhanced-yellow-fluorescent-protein-tagged TrkA (TrkA-EYFP) was then visualized in primary cultures of DRG neurons of each genotype. 24 hr after transduction of the adenoviral vector, the cells were observed using a fluorescence microscope equipped with a cooled electron-multiplying charge-coupled device (EM-CCD) camera at four frames per second. As a result, the motility of TrkA(+) vesicles (Movie S1) was significantly altered in the *Kif1a*$^{+/-}$ DRG neurons, as quantified with kymographs (Figure 5A). The numbers of anterogradely and retrogradely moving vesicles carrying TrkA counted over a period of 1 min were decreased in the *Kif1a*$^{+/-}$ axons (Figure 5B). Because those of transported mitochondria remained unchanged (Figure 5B; Movie S2) and because our preliminary data suggested that the TrkA(+) accumulations in the *Kif1a*$^{+/-}$ axons did not largely contain mitochondria (data not shown), this bidirectional reduction in TrkA transport was considered to be specific.

The motility of TrkA vesicles was then analyzed in detail. More than 500 motile segments from three independent axons were traced from the kymographs. The run length of TrkA vesicles in *Kif1a*$^{+/-}$ axons was only significantly decreased in the anterograde direction (Figure 5C). Classification of the initial direction of more than 100 vesicles each in the observation period revealed the existence of significantly more stationary vesicles and a decrease in the ratio of anterograde transport in *Kif1a*$^{+/-}$ axons (Figure 5D). As for directionality changes, the mean interval of anterograde-to-retrograde changing was 58 s (61 times in 3,548 s) in *Kif1a*$^{+/+}$ axons and 31 s (62 times in 1,912 s) in *Kif1a*$^{+/-}$ axons, but that of retrograde-to-anterograde changing was 48 s (56 times in 2,707 s) in *Kif1a*$^{+/+}$ axons and 56 s (62 times in 3,475 s) in *Kif1a*$^{+/-}$ axons, suggesting that the anterograde TrkA vesicles tended to more frequently return in *Kif1a*$^{+/-}$ axons. Finally, the segmental velocity histogram especially revealed a significant decrease in normalized segment number for the fast (>1.0 μm/s) anterograde component (Figure 5E, arrow).

These results demonstrate that anterograde but not retrograde TrkA vesicles stopped sooner, paused for longer periods of time, and frequently returned in *Kif1a*$^{+/-}$ neurons, suggesting that anterograde processivity of TrkA vesicles was specifically disorganized by KIF1A deficiency.

TrkA Is Transported by a KIF1A/Rab3-Based System

To further demonstrate that TrkA is a cargo for KIF1A, vesicle immunoprecipitation (IP) experiments were performed using axonal vesicles fractionated from the cauda equina to determine the role of KIF1A in TrkA axonal transport. KIF1A-carrying vesicles were purified from axonal vesicles using a specific antibody, and TrkA was co-immunoprecipitated with them (Figure 6A). Then, TrkA-carrying vesicles were purified in the same way, and KIF1A, but not the KIF5B motor, was co-immunoprecipitated as well, suggesting that TrkA and KIF1A are specifically associated with the same axonal vesicles (Figure 6B).

To characterize the identity of these TrkA/KIF1A-associated vesicles, we performed tag-immunoprecipitation (TagIP) using TRPV1-F11 cell lysates to examine the TrkA-GTP-Rab3

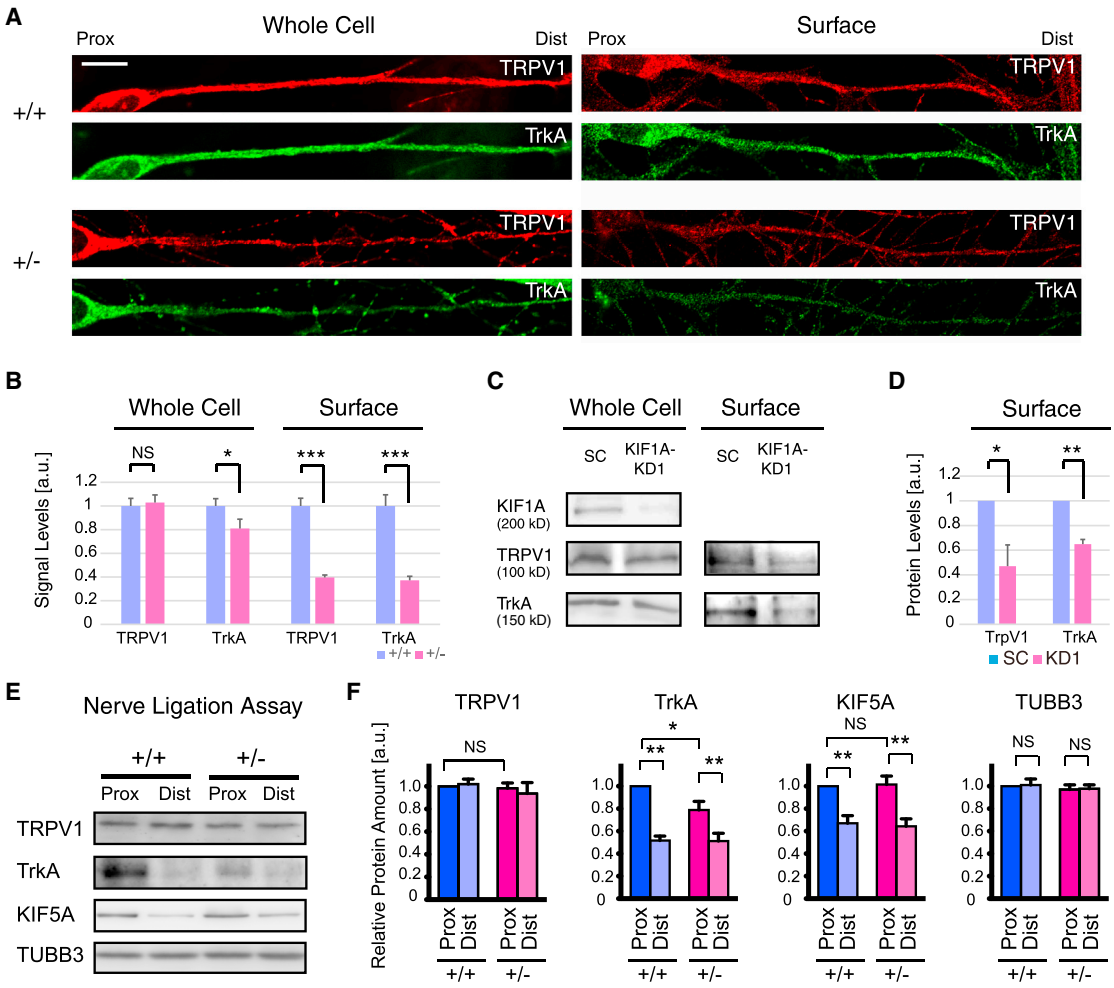


Figure 4. Differential Phenotypes of TRPV1 and TrkA Distributions in *Kif1a*$^{+/-}$ Neurons

(A and B) Immunocytochemistry of DRG neurons of the indicated genotypes for TRPV1 and TrkA with (Whole Cell) or without (Surface) permeabilization (A) and the corresponding quantification (B). Note that KIF1A deficiency reduced the surface expression of both proteins but only the cytoplasmic expression of TrkA. NS, $p > 0.05$; *$p < 0.05$; ***$p < 0.001$; Welch's t test. n = 18–30. Error bars show SEM. Scale bar, 10 μm.

(C and D) Surface biotinylation assay of TRPV1-F11 cells transduced with mock (SC) and KIF1A (KD1) miRNA knockdown vectors and immunoblotted for KIF1A, TRPV1, and TrkA (C). The quantification is shown in (D). Note that KIF1A depletion significantly reduced the levels of TrkA and TRPV1 on the cell surface but not in the whole cells. *$p < 0.05$; **$p < 0.01$; Welch's t test. n = 3–6. Error bars show SEM.

(E and F) Nerve ligation assay. Immunoblotting of lysates from 5-mm regions of nerve collected proximal and distal to the ligation sites after 5 hr. The immunoblotting (E) is for TRPV1, TrkA, KIF5A, and β3-tubulin (TUBB3), and the statistical analysis (F) is of five independent experiments normalized by the average amount of proximally accumulated proteins in *Kif1a*$^{+/+}$ mice. The values are plotted as the mean ± SEM. NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; Student's t test.

association (Figure 6C). The results suggested that the adaptor Rab3QL tagged with TagRFP was specifically coprecipitated with TrkA-ECFP but not with the ECFP-only negative control. In a comigration assay (Figures 6D–6F), TagRFP-tagged Rab3QL nicely colocalized and comigrated with TrkA-ECFP- and KIF1A-EYFP-containing transporting vesicles (Figures 6D and 6E; Movie S3). In this condition, most of the TrkA-transporting vesicles contained KIF1A and Rab3QL (Figure 6F). Because the QL mutation mimicked the active and transportable GTP form of Rab3, these data suggested that TrkA-containing vesicles associated with KIF1A are transported by the KIF1A and GTP-Rab3 system in parallel with synaptic vesicle precursors (Niwa et al., 2008).

We further performed triple immunofluorescence labeling for TrkA, KIF1A, and the endosome marker APPL1 of *Kif1a*$^{+/+}$ DRG neuronal axons using the semi-superresolution Airyscan detector (Figures 6G–6I). As a result, TrkA immunofluorescence signals partially colocalized with either KIF1A immunofluorescence signals or APPL1 immunofluorescence signals. However, the tendency for the TrkA signal to be associated with each of these signals was statistically independent in a reproducible manner (n = 228, $p > 0.05$, chi-square test), suggesting that the anterogradely transported KIF1A-associated TrkA vesicles are potentially distinct from the retrograde signaling endosomes.

Finally, we assessed the relationship between KIF1A and its closely related motor KIF1Bβ. Immunofluorescence microscopy

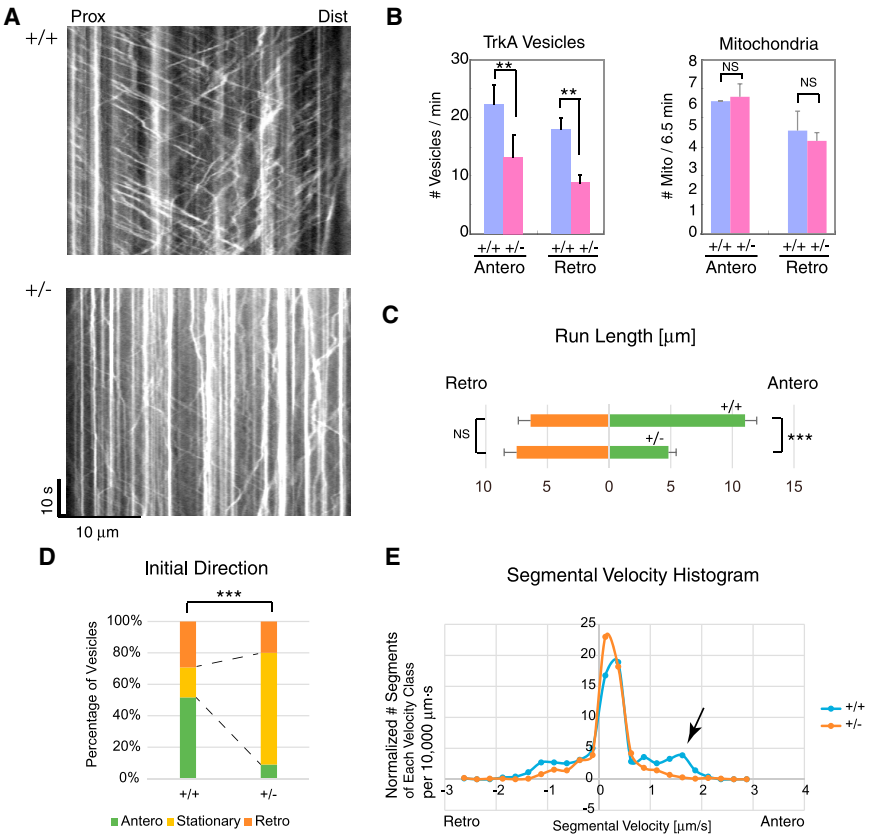


Figure 5. Axonal Transport of TrkA

(A) Representative kymographs of TrkA-EYFP vesicle movements in axons in dissociated cultures of *Kif1a*$^{+/+}$ and *Kif1a*$^{+/-}$ DRG neurons. See also Movie S1.

(B) Number of TrkA-EYFP vesicles or mitochondria (Movie S2) that exited (Antero) or entered (Retro) the initial axon segments of DRG neurons. NS, $p > 0.05$; **$p < 0.01$; Student's t test; n = 3–7 neurons from three *Kif1a*$^{+/+}$ and three *Kif1a*$^{+/-}$ mice. Error bars show SD (left) and SEM (right).

(C–E) Axonal transport parameters of TrkA vesicles in cultured DRG neurons of the indicated genotypes.

(C) Run length of motile vesicles in the indicated directions. NS, $p > 0.05$; ***$p < 0.001$; Student's t test; n = 130–185 segments. Error bars show SEM.

(D) Initial direction of the vesicles at the start of observation. ***$p < 0.001$, Fisher's exact probability test, n = 224.

(E) Histogram for segmental velocities normalized by the total area of kymographs. The arrow indicates the fast anterograde component that was significantly decreased by KIF1A deficiency.

of cultured DRG neurons revealed that KIF1Bβ was only expressed in the very early stages after plating (Figures S2A and S2B), consistent with its general developmental expression pattern (Gumy et al., 2011; Takemura et al., 1996). Furthermore, vesicle IP experiments with adult brain lysates revealed that only KIF1A and not KIF1Bβ was specifically associated with TrkA-containing vesicles (Figure S2C). These results suggest that TrkA transport is a specific function of the KIF1A motor, which may partly explain the adult onset of KIF1A deficiency-associated sensory neuropathy.

NGF-TrkA Signaling Is Attenuated in *Kif1a*$^{+/-}$ DRG Neurons

Our results suggested that NGF/TrkA signaling could be altered in *Kif1a*$^{+/-}$ DRG neurons. It is well established that TrkA signaling is required for the survival and function of sensory neurons (Crowley et al., 1994; Huang and Reichardt, 2003; Levi-Montalcini and Angeletti, 1963; Zhang et al., 2005). When NGF binds to TrkA on the plasma membrane, the NGF/TrkA complex is internalized into signaling endosomes to activate Ras/PI3K signaling, and Akt is phosphorylated (Klein et al., 1991; Ohmichi et al., 1992; Soltoff et al., 1992; Thomas et al., 1992). Therefore, we compared the extent of the internalization of NGF-biotin in a knockdown system of TRPV1-F11 cells (Figures 7A and 7B). After overnight serum starvation in the presence of an anti-NGF antibody, the knockdown or control cells were treated with 40 ng/ml mouse NGF 2.5S-biotin for 6 hr and subjected to SDS-PAGE. As a result, the level of internalized NGF-biotin was significantly decreased in KIF1A-KD cells to half that in the control, indicating the impairment of NGF reception by KIF1A deficiency.

We further tested the levels of NGF/TrkA-mediated PI3K signaling using this KIF1A knockdown system. We transduced cells with one of two different types of knockdown vectors (KD1 and KD2) as well as with an EGFP-tagged constitutively active Ras expression vector (RasCA-EGFP) or a scrambled miRNA vector (SC) and stimulated them with NGF for 1 hr before subjecting them to immunoblotting (Figures 7C and 7D). In the control cells, 1 hr of NGF treatment significantly enhanced Akt phosphorylation, which is a marker for PI3K signaling, although the level of total Akt and that of α-tubulin remained largely unchanged. The two knockdown vectors similarly reduced the expression of KIF1A, and the NGF-mediated increase in Akt phosphorylation levels was significantly impaired in a similar and consistent manner. Furthermore, these reductions in Akt activation were nicely rescued by RasCA-EGFP expression, confirming that KIF1A deficiency reversibly affected the reception of NGF signaling. These results collectively demonstrated that, in *Kif1a*$^{+/-}$ DRG neurons, the TrkA/PI3K signaling pathway was hyposensitive to NGF.

In this assay, we also noticed that KIF1A expression in wild-type cells was upregulated 1.5-fold after 1 hr of NGF stimulation (Figures 7C and 7D). According to RT-PCR normalized to *GAPDH* cDNA, a statistically significant, approximately 1.2-fold upregulation of *Kif1a* cDNA was detected (Figure 7E, left). A similar upregulation was detected when the PI3K pathway was directly stimulated using the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) inhibitor SF1670 (250 nM overnight; Figure 7E, right; Li et al., 2011), suggesting the existence of a positive feedback regulation between KIF1A

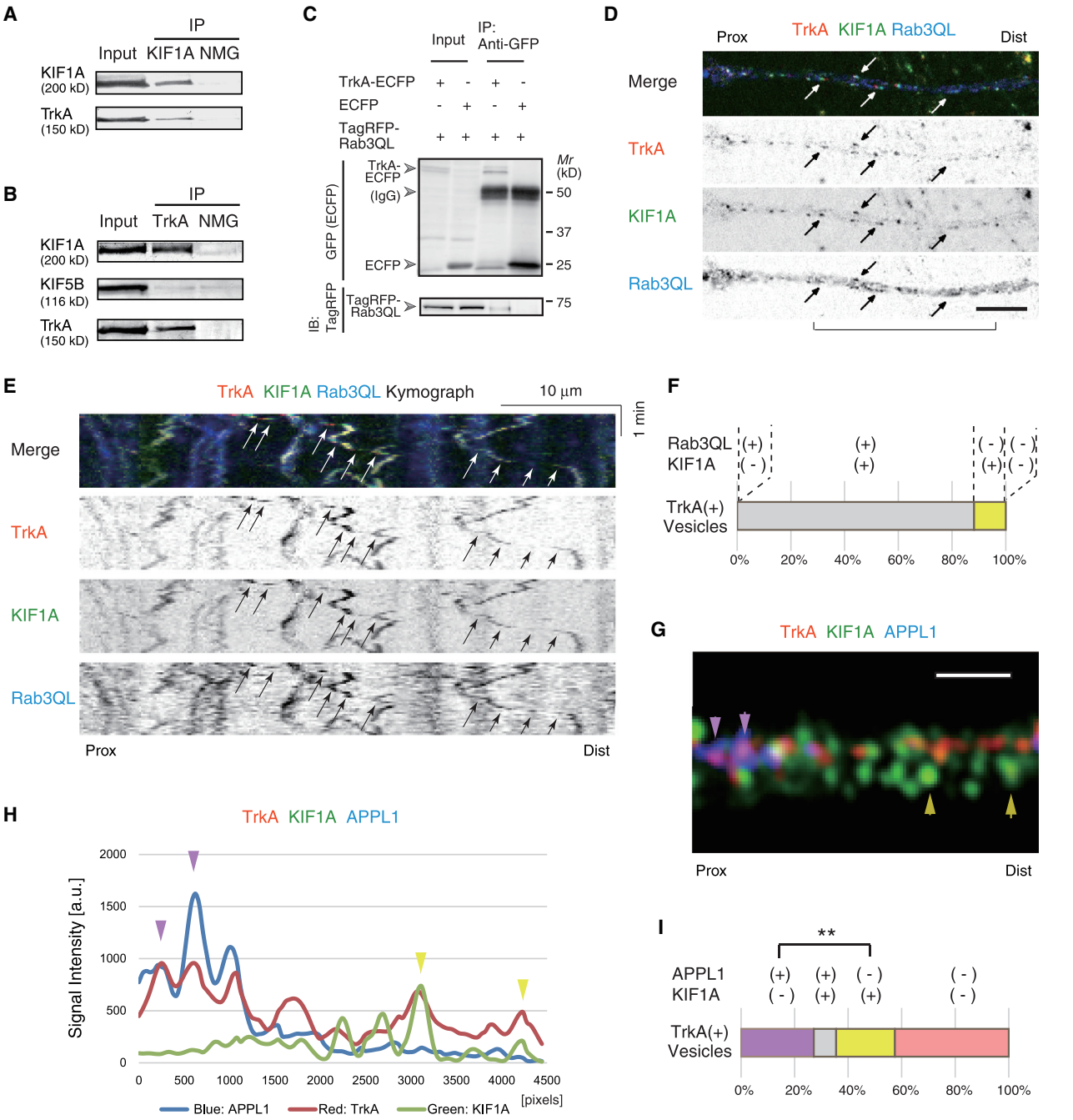


Figure 6. Transport of TrkA by GTP-Rab3-Containing KIF1A Vesicles

(A and B) Vesicle IP from the cauda equina of *Kif1a*$^{+/+}$ mice using anti-KIF1A (A) and anti-TrkA (B) antibodies or normal mouse IgG (NMG) and probed with the indicated antibodies. Note that TrkA was co-immunoprecipitated with KIF1A but not with KIF5B.

(C) TagIP. Immunoprecipitates against ECFP tags were immunoblotted (IB) against ECFP and TagRFP from lysates of TRPV1-F11 cells expressing TrkA-ECFP and TagRFP-Rab3QL and ECFP and TagRFP-Rab3QL, respectively. Note that TrkA-ECFP, but not ECFP only, specifically coprecipitated with TagRFP-Rab3QL. These IPs were reproduced multiple times. IB, immunoblot. Related to Figure S2.

(D–F) Comigration of TrkA-ECFP, KIF1A-EYFP, and TagRFP-Rab3QL in a DRG neuronal axon represented by initial micrograph (D, related to Movie S3) and kymograph (E) and quantification of the colocalizing population in anterograde TrkA(+) vesicles (F). n = 17. The bracket in (D) indicates the segment for the kymograph. Note that triple-labeled vesicles (arrows) are processively transported down the axons. Scale bars, 10 μm.

(G–I) Triple-labeled immunocytochemistry (G) of a DRG neuron at day in vitro (DIV) 14 for APPL1 (blue), KIF1A (green), and TrkA (red) with a line profile (H) and quantification of the colocalizing population in TrkA(+) vesicles (I). The images were obtained with a ZEISS Airyscan device. Points of TrkA/APPL1 (purple arrows) and TrkA/KIF1A (yellow arrows) colocalization are indicated. Scale bar, 1 μm. **p < 0.01, chi-square test. n = 228.

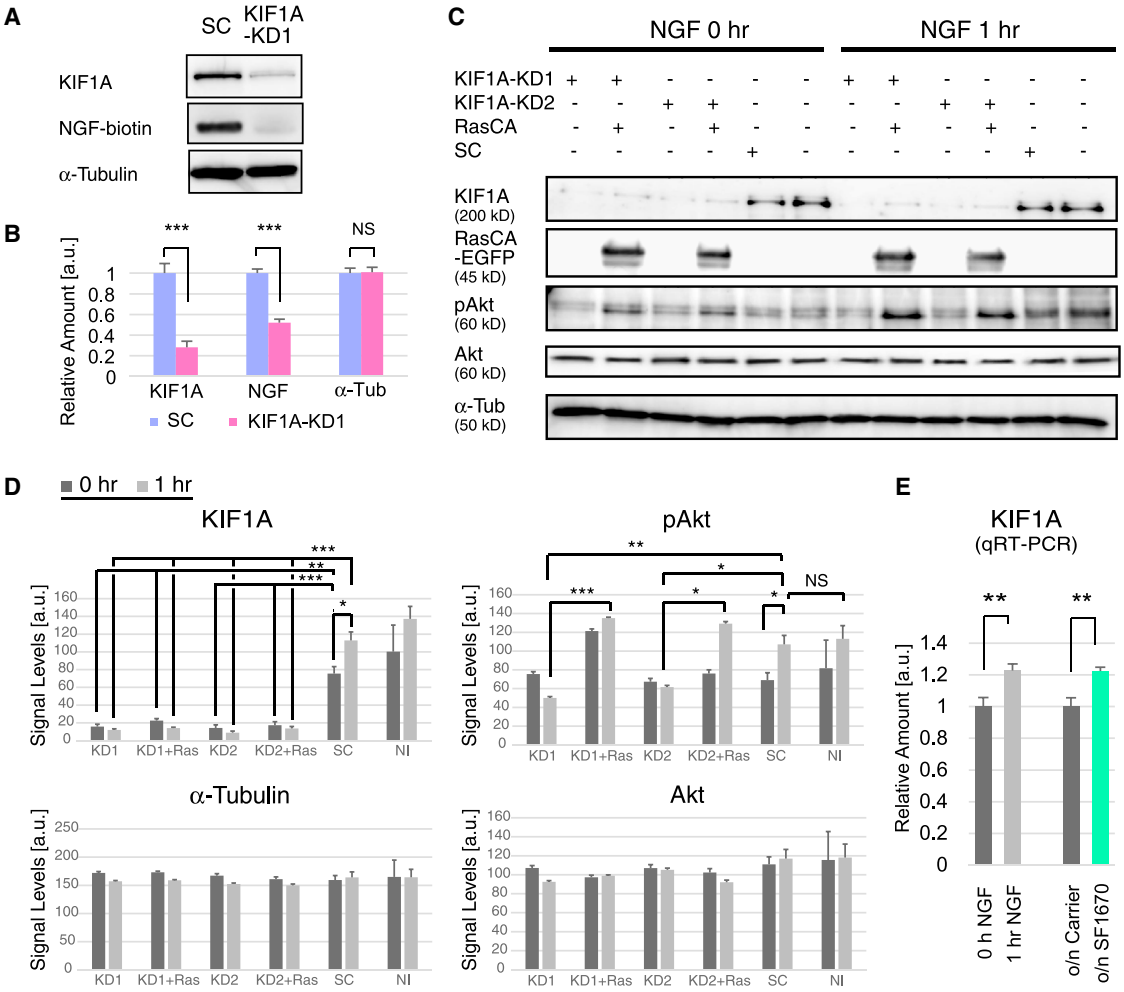


Figure 7. Impairment of the NGF/TrkA Signaling Pathway in *Kif1a*$^{+/-}$ DRG Neurons

(A and B) NGF-biotin internalization assay (A) and its quantification (B). TRPV1-F11 cells that were transduced with scrambled or KIF1A-KD1 miRNA were incubated with NGF-biotin for 6 hr and subjected to immunoblotting with the indicated antibodies or avidin-horseradish peroxidase (HRP) (for NGF-biotin). Note that NGF internalization was significantly impaired by KIF1A deficiency. NS, $p > 0.05$; ***$p < 0.001$; Welch's t test; n = 6. Error bars show SEM.

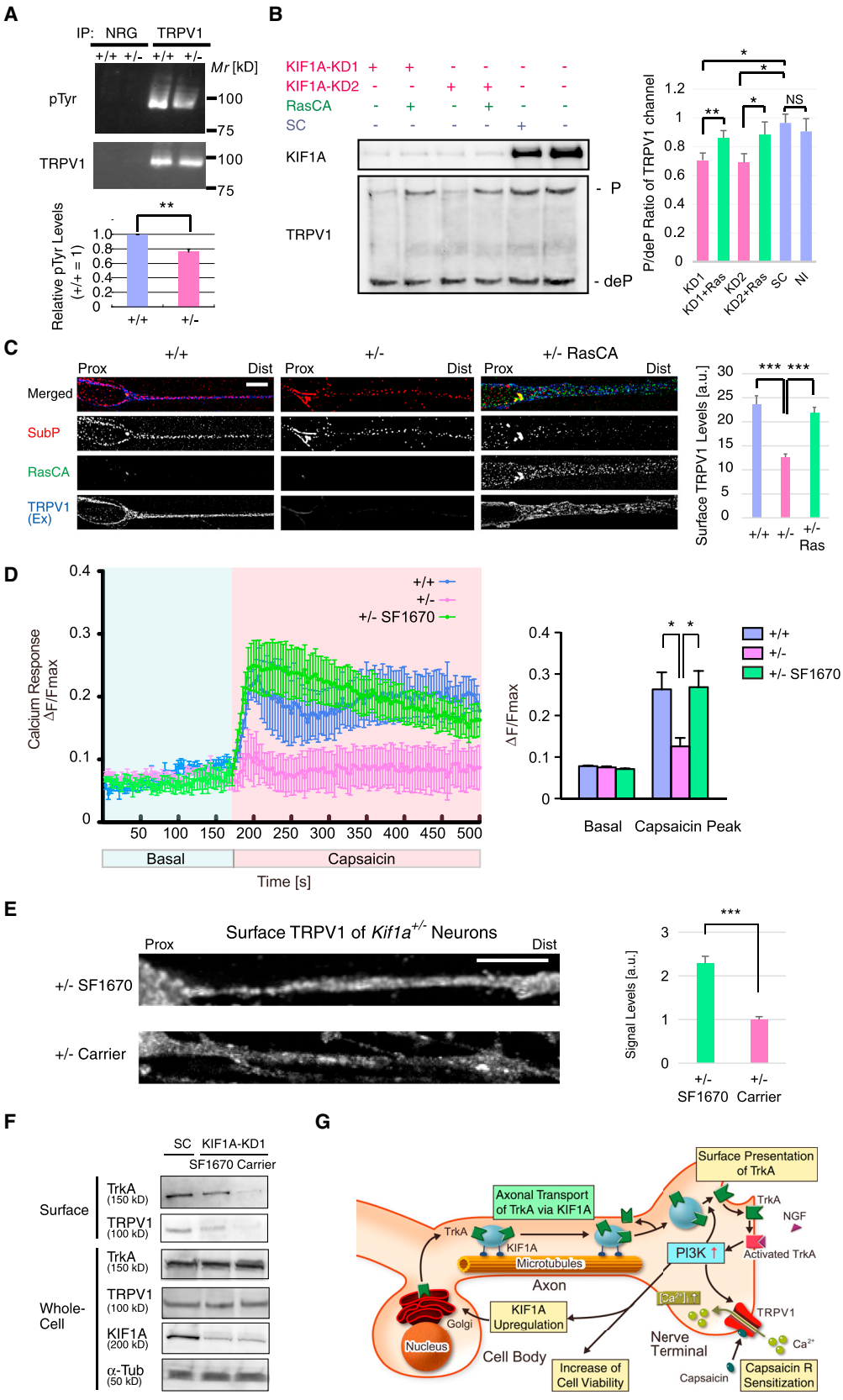
(C and D) In vitro NGF signaling assay in TRPV1-F11 cells with knockdown using KIF1A (KD1 or KD2) or scrambled negative control miRNAs with or without rescue with RasCA-EGFP. Labeling was performed with anti-KIF1A, -GFP, -pAkt, -total Akt, and -α-tubulin antibodies (C) and was quantified (D). NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; Welch's t test. n = 3–6. Error bars show SEM.

(E) qRT-PCR assay of mouse *Kif1a* cDNA in TRPV1-F11 cells before (0 hr) and after (1 hr) NGF stimulation or overnight starvation with treatment with the carrier (0.1% DMSO) or SF1670 (250 nM) and normalized with *GAPDH*. **$p < 0.01$, Welch's t test, n = 8. Error bars show SEM.

expression and TrkA/PI3K signaling. This may exaggerate the consequence of a subtle change in motor activity. This result was also consistent with our previous results showing that the brain-derived neurotrophic factor (BDNF)-mediated signaling enhances *Kif1a* transcription (Kondo et al., 2012).

PI3K-Dependent TRPV1 Phosphorylation in *Kif1a*$^{+/-}$ DRGs

Previous studies have shown that the sensitivity of TRPV1, a capsaicin receptor, is augmented by its tyrosine-phosphorylation due to NGF/TrkA/PI3K signaling (Amaya et al., 2004; Ji et al., 2002; Xue et al., 2007; Zhang et al., 2005), which could result in the altered cellular response. To test this hypothesis, TRPV1 phosphorylation was analyzed both with immunoprecipitation and Phos-tag immunoblotting. First, TRPV1 was immunoprecipitated from DRG neurons from 9-month-old *Kif1a*$^{+/+}$ and *Kif1a*$^{+/-}$ mice. After the results were normalized to the TRPV1 levels, immunoblotting against phosphotyrosine demonstrated that TRPV1 tyrosine phosphorylation was significantly reduced in the *Kif1a*$^{+/-}$ DRG neurons (Figure 8A; approximately 24% reduction in *Kif1a*$^{+/-}$ samples). Then we directly subjected the lysates of the knockdown rescue system to Phos-tag SDS-PAGE electrophoresis to separate the signals according to the level of phosphorylation (Figure 8B). The phosphorylated forms of TRPV1, which move slower through the gel, were significantly reduced in the KIF1A knockdown cells. Furthermore, RasCA-EGFP significantly restored the phosphorylation levels, suggesting that this downregulation of TRPV1 phosphorylation was due



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to specific downregulation of TrkA/Ras signaling. These data suggest that KIF1A reduction affected the Ras-mediated tyrosine phosphorylation level of the TRPV1 channel both in vivo and in vitro.

It has been demonstrated that this tyrosine phosphorylation facilitates the surface presentation of the TRPV1 protein (Zhang et al., 2005). Accordingly, we immunolabeled the cell surface TRPV1 channels in primary DRG neurons (Figure 8C). As a result, in the SubP-positive *Kif1a*$^{+/-}$ nociceptive neurons, which are largely consistent with the TrkA(+) population (Basbaum and Woolf, 1999), a significant reduction in the level of surface TRPV1 was revealed, which was rescued by RasCA-EGFP transduction.

Finally, we investigated whether facilitation of PI3K signaling rescued the phenotype of the KIF1A-deficient neurons as direct evidence that PI3K signaling is essential and sufficient for KIF1A-mediated augmentation of capsaicin sensitivity. Overnight treatment with SF1670 (250 nM) significantly enhanced the $[Ca]_i$ capsaicin response of *Kif1a*$^{+/-}$ DRG neurons, exceeding that of *Kif1a*$^{+/+}$ neurons (Figure 8D). Furthermore, the surface expression of TRPV1 in the *Kif1a*$^{+/-}$ DRG neurons (Figure 8E) and that of the KIF1A knockdown TRPV1-F11 cells (Figure 8F) were significantly rescued. Interestingly, the surface presentation of TrkA in the knockdown cells was also partly rescued by SF1670 (Figure 8F). This suggested the existence of another positive feedback system enhancing the surface presentation of the TrkA receptor itself by its downstream PI3K signaling because PI3K/Akt/14-3-3-mediated enhancement of the surface presentation of membrane proteins in general has been reported previously in other systems (Chung et al., 2009). Therefore, the altered capsaicin response in the *Kif1a*$^{+/-}$ DRG neurons was assumed to be primarily due to alterations in TRPV1 phosphorylation and its surface presentation, which is dependent on KIF1A-mediated NGF/TrkA/Ras/PI3K signaling. Because PI3K signaling also supports cell survival and augments *Kif1a* gene transcription, KIF1A is proposed to be a synergistic regulator of the sensory function of TrkA(+) neurons in the DRG (Figure 8G).

DISCUSSION

Sensory Phenotypes of the *Kif1a*$^{+/-}$ Mice

Previous studies have assumed that KIF1A is the exclusive motor for synaptic vesicle precursors (Hall and Hedgecock, 1991; Niwa et al., 2008; Okada et al., 1995; Wagner et al., 2009; Yonekawa et al., 1998). In addition, *Kif1a*$^{-/-}$ mice were shown to die shortly after birth, with synaptic vesicle defects and neuronal cell death in the CNS (Yonekawa et al., 1998), which has made it difficult to further analyze KIF1A function in vivo. The results from this study showed that KIF1A-haploinsufficient mice exhibited sensory neuronal phenotypes (Figure 1); two major changes were observed in the sensory neurons. First, the number of TrkA(+) neurons was specifically and progressively reduced, possibly by apoptosis, in the *Kif1a*$^{+/-}$ mice (Figure 2). The juvenile- or regenerating-only expression of the related motor KIF1Bβ in the DRG (Figure S2; Gumy et al., 2011; Takemura et al., 1996) also explains why the *Kif1a*$^{+/-}$ phenotype was adult-onset. Second, the TRPV1-dependent capsaicin response was significantly affected in surviving DRG nociceptive neurons (Figure 3), suggesting that the sensitivity of TRPV1, which is a capsaicin receptor in DRG neurons, was impaired. Capsaicin and other nociceptive signals activate TRPV1 (Tominaga et al., 1998), and alterations of this receptor also result in sensory neuronal phenotypes such as those observed in *Trpv1*$^{-/-}$ and *Pirt*$^{-/-}$ mice (Caterina et al., 2000; Kim et al., 2008). Taken together, these abnormalities are thought to be responsible for the sensory phenotypes observed in the KIF1A-haploinsufficient mice (Figure 8G).

Molecular Mechanisms that Disrupt the Function of *Kif1a*$^{+/-}$ Sensory Neurons

Because KIF1A transports synaptic vesicle precursors, a reduction in synaptic vesicles could partially explain the sensory phenotypes. However, the Ca and electrophysiological capsaicin responses (Figure 3) were recorded directly from each neuron, suggesting that the present phenotype is more likely cell-autonomous. In addition, previous genetic studies have shown that defective synaptic vesicles are not related to

Figure 8. Augmentation of Ras/PI3K Signaling Rescues the KIF1A Phenotypes

(A and B) TRPV1 phosphorylation.

(A) Black and white reversal immunoblots depicting the level of tyrosine phosphorylation of TRPV1 channel in DRG lysates of the indicated genotypes. Immunoprecipitation was performed against TRPV1, and immunoblotting was performed against phospho-Tyrosine (pTyr) and TRPV1. **$p < 0.01$, Student's t test.

(B) Phos-tag-mediated immunoblotting against KIF1A and TRPV1 in TRPV1-F11 cells transduced with the indicated constructs with the quantification. NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; Welch's t test. Error bars show SEM.

(C) Superresolution structure illumination microscopy (SR-SIM) images of immunocytochemical surface labeling of TRPV1 in DRG neurons with or without rescue by RasCA-EGFP expression. Counterstaining for SubP was used to identify nociceptive neurons, and the quantification is shown. Scale bar, 5 μm. ***$p < 0.001$, Welch's t test, n = 17–28. Note that the surface presentation of TRPV1 channels in *Kif1a*$^{+/-}$ neurons can be restored by Ras-mediated signal transduction. Error bars show SEM.

(D–F) Rescue of *Kif1a*$^{+/-}$ neurons by direct stimulation of the PI3K signaling pathway.

(D) Capsaicin-induced Ca transients in SF1670-treated *Kif1a*$^{+/-}$ DRG neurons and the corresponding quantification. *$p < 0.05$, one-way ANOVA. Error bars show SEM. n = 11–16 from 3 mice. +/+ and +/− data are reproduced from Figure 3A.

(E) Surface TRPV1 immunofluorescence of *Kif1a*$^{+/-}$ DRG neurons treated with SF1670 or 0.1% DMSO (Carrier) and the corresponding quantification. Scale bar, 10 μm. ***$p < 0.001$, Welch's t test. Error bars show SEM. n = 17–25.

(F) Immunoblotting of a surface biotinylation assay of TRPV1-F11 cells transduced with scrambled or KIF1A knockdown miRNA and treated with SF1670 or carrier, respectively, using the indicated antibodies.

(G) KIF1A-mediated axonal transport of the NGF receptor TrkA is essential for PI3K signaling in DRG neurons, which enhances the nociceptive function of peripheral nerves through sensitization of the capsaicin receptor, surface presentation of TrkA receptor, an increase in cell viability, and an enhancement of *Kif1a* transcription.

neuronal viability (Geppert et al., 1997; McMahon et al., 1996; Takei et al., 1995). This study demonstrated that the axonal transport and cell surface distribution of TrkA were disrupted in *Kif1a*$^{+/-}$ DRG neurons and in KIF1A knockdown TRPV1-F11 cells (Figure 4). The motility of TrkA-EYFP vesicles was also altered (Figure 5). The number of moving TrkA(+) vesicles was significantly reduced in *Kif1a*$^{+/-}$ axons, and TrkA(+) anterograde vesicles tended to translocate more slowly, stop more often, and more frequently change direction. Because the mislocalization and abnormal motility of cargos in motor protein-mutant animals is a well-established means of evaluating the cargo-motor relationship (Barkus et al., 2008; Hall and Hedgecock, 1991; Niwa et al., 2008; Tanaka et al., 1998; Zahn et al., 2004), our results strongly suggest that TrkA is a cargo for KIF1A.

Furthermore, TrkA co-immunoprecipitated with KIF1A and GTP-Rab3, and these proteins comigrated in DRG axons (Figure 6; Movie S3). Indeed, the *Kif1a*$^{+/-}$ neurons were hyposensitive to NGF, which functions as the major TrkA ligand (Figure 7). This result was further supported by the observation that the number of TrkA(+) and NGF-responsive neurons was specifically reduced in *Kif1a*$^{+/-}$ mice (Figure 2) and that TRPV1 phosphorylation and surface expression were reduced in surviving *Kif1a*$^{+/-}$ DRG neurons (Figures 4 and 8), although the axonal distribution of TRPV1 appeared to be unchanged (Figure 4). NGF/TrkA signaling has been shown to support the survival of sensory neurons (Crowley et al., 1994; Huang and Reichardt, 2003; Levi-Montalcini and Angeletti, 1963) and to stimulate TRPV1 expression and phosphorylation (Amaya et al., 2004; Chuang et al., 2001; Ji et al., 2002; Xue et al., 2007; Zhang et al., 2005). Because receptor tyrosine kinase signaling and activity-dependent Ca signaling synergistically stimulate the cell survival transcriptional factor MEF2 in neurons (Mao et al., 1999; Wiedmann et al., 2005), this result is consistent with previous finding that glutamate stimulation could rescue the viability of *Kif1a*$^{-/-}$ neurons (Yonekawa et al., 1998). These previous studies are consistent with this study, which demonstrated a decreased number of TrkA neurons and a weakened response of *Kif1a*$^{+/-}$ sensory neurons to capsaicin stimulation (Figure 8G).

Interestingly, anterograde as well as retrograde transport of TrkA was reduced in *Kif1a*$^{+/-}$ axons (Figure 5B). This observation appears to be inconsistent with previous results demonstrating that KIF1A acts as a motor for anterograde axonal transport (Okada et al., 1995). A possible explanation for this apparent discrepancy is that the amount of TrkA that was anterogradely transported from cell bodies to axon terminals was reduced and that the amount of TrkA transported back to the cell bodies should consequently be reduced. Another possibility is that anterograde and retrograde machineries could support each other, as shown in recent studies (Ally et al., 2009; Barkus et al., 2008). Although these two possibilities are not mutually exclusive, our vesicle motility analyses further revealed that KIF1A mainly contributes to the anterograde but not retrograde processivity of TrkA vesicle motility. This was consistent with previous results showing that an increase in the molar ratio of KIF1A/Unc104 motor per cargo enhances the anterograde processivity because of the increased capacity to form ''functional dimers'' (Okada et al., 2003; Rashid et al., 2005). Thus it is much likely that the anterograde transport is the primary point of defects. Future studies are needed to determine the precise mechanisms involved in the decrease in retrograde transport of TrkA following the loss of KIF1A.

To date, the candidate genes and pathogenesis of hereditary sensory neuropathies are still largely elusive (Verhoeven et al., 2006). The *NGFB* gene, which encodes for the NGF precursor protein, results in hereditary sensory neuropathy type V (HSN5) (Einarsdottir et al., 2004). In addition, the *TRKA* gene is the cause of congenital insensitivity to pain with anhidrosis (CIPA) (Indo et al., 1996). Recently a *KIF1A* point mutation has been reported to be responsible for hereditary sensory and autonomic neuropathy type 2 (HSANII) (Rivière et al., 2011). Collectively, knowledge of the causes of these human genetic disorders will further support our hypothesis that the KIF1A-mediated NGF/TrkA/PI3K signaling pathway is responsible for sensory function in the peripheral nervous system.

EXPERIMENTAL PROCEDURES

Mice and Cells

Kif1a conditional knockout mice were generated (Figure S1), crossed with *CAG-Cre* female mice (Sakai and Miyazaki, 1997), and maintained in a specific pathogen-free environment at the University of Tokyo in accordance with institutional guidelines. The behavioral tests (Crawley, 2007; Fuchs et al., 1999; Gonzales-Rios et al., 1986; O'Callaghan and Holtzman, 1975; Pitcher et al., 1999; Shibata et al., 1989), primary culture of mouse DRGs (Okabe and Hirokawa, 1991), and routine culture of TRPV1-F11 cells (Nakanishi et al., 2010) were performed as described previously. For physiological recordings, dissociated DRG neurons were subjected to Ca imaging using Fluo-4AM (Thermo Fisher Scientific) (Tanaka et al., 2005) and whole-cell patch-clamp (Zhang et al., 1998) basically as described previously. Only cells with a positive response to capsaicin were included in the statistics.

Antibodies and Vectors

Anti-KIF1A antibodies (RRID: AB_2314702) were raised with rabbits immunized with the synthetic peptides CRRSAQMRV (C-terminal) and CMDSGPNKNKKKKK (K-loop). The anti-KIF5A (RRID: AB_2571744), anti-KIF5B (RRID: AB_2571745), and anti-KIF1Bβ (RRID: AB_2571746) antibodies have been described previously (Kanai et al., 2004; Zhao et al., 2001). The rabbit anti-TrkA antibody was a gift from Prof. Lou Reichardt (University of California, San Francisco, RRID: AB_2315490). Other antibodies were purchased as described in the Supplemental Experimental Procedures. Expression and knockdown vectors were generated using the ViraPower adenoviral expression system (Invitrogen) as described previously (Nakata et al., 1998; Niwa et al., 2008; Yang et al., 2014).

Microscopy

Immunohistochemistry and immunocytochemistry were performed basically as described previously (Ueno et al., 2011; Zhou et al., 2009). These experiments were reproduced more than twice, and the images were analyzed using GNU image manipulation program (GIMP) version 2.6 (http://www.gimp.org/), MetaMorph version 7.7.8.0 (Molecular Devices), and/or ImageJ version 1.49 (NIH, http://rsb.info.nih.gov/nih-image/) software.

Biochemistry

Immunoblotting was performed using electrochemiluminescence (ECL, GE Healthcare) as described previously (Yang et al., 2014). For the NGF internalization assay and in vitro NGF signaling assay, overnight-starved cells were treated with 40 ng/ml mouse NGF 2.5S-Biotin (N-240-B, Alomone Labs) or 50 ng/ml mouse 2.5S-NGF (BD Biosciences, 354005). The nerve ligation experiment was conducted as described previously (Hirokawa et al., 1990). Cell surface proteins were isolated using a Pierce cell surface protein isolation kit (Thermo Scientific). For vesicle immunoprecipitation, mouse cauda equina vesicles eluted with internal medium (Okada et al., 1995), and mouse brain

lysates eluted with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-sucrose buffer were precipitated with the μMACS system (Miltenyi Biotec). TagIP (Yang et al., 2014), the TRPV1 tyrosine phosphorylation assay (Zhang et al., 2005), and Phos-tag (Wako) SDS-PAGE immunoblotting (Kinoshita et al., 2006) were performed as described previously. These assays were reproduced more than twice and subjected to quantification using GIMP and ImageJ software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and three movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.05.002.

AUTHOR CONTRIBUTIONS

N.H. conceived and directed the project. Y.T., S.N., and M.D. conducted experiments. A.F., L.W., and R.Z. partly conducted the revising experiments. Y.T., S.N., M.D., A.F., L.W., R.Z., and N.H. discussed the data. Y.T., S.N., M.D., and N.H. wrote the manuscript.

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